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Measurement of the second order non-linear susceptibility of collagen using polarization modulation and phase-sensitive detection

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ABSTRACT

The measurement of the second order nonlinear susceptibility of collagen in various biological tissues has potential applications in the detection of structural changes which are related to different pathological conditions. We investigate second harmonic generation in rat-tail tendon, a highly organized collagen structure consisting of parallel fibers. Using an electro-optic modulator and a quarter-wave plate, we modulate the linear polarization of an ultra-short pulse laser beam that is used to measure second harmonic generation (SHG) in a confocal microscopy setup. Phase-sensitive detection of the generated signal, coupled with a simple model of the collagen protein structures, allows us to measure a parameter γ related to nonlinear susceptibility and to determine the relative orientation of the structures. Our preliminary results indicate that it may be possible to use this parameter to characterize the structure.

Keywords: second harmonic generation, collagen, phase-sensitive detection, polarization modulation

1. INTRODUCTION

Collagen is a structural protein comprising more than twenty genetically distinct types and is found in many tissues such as skin, cartilage, tendon and bone. The collagen molecule is a rigid triple-helical structure with a diameter of a few nanometers and a length of a few hundred nanometers; it is often organized into fibrils, which are less than a micrometer in diameter but a few hundred micrometers in length[1,2,3]. These fibrils are themselves arranged in macroscopic bundles called fibers; collagen fibers are found throughout connective tissues. Understanding the structure of collagen and detecting changes in its organization on these different scales is important because it might lead to new diagnostic techniques for certain pathological conditions such as abnormal wound healing, glyco-oxidative damage, and superficial tumors; it is also important in the development of artificial tissues. Currently, there are no rapid, non-invasive techniques for detecting changes in collagen structure on microscopic scales.

Confocal microscopy of optical second harmonic generation (SHG) offers a possible solution. The second-order nonlinear polarizability of collagen molecules and fibrils and the non-centrosymmetric structures they form leads to efficient SHG. Both macroscopic and microscopic organization of collagen molecules contributes to enhancing SHG. Collagen fibers are typically 1 to 15 μm in diameter and millimeters long; they are the most organized constituents in connective tissue such as skin and tendon. Because substantial second harmonic light is generated only in organized structures that lack inversion symmetry, SHG is inherently sensitive to collagen structure and not to the surrounding tissue which is disorganized on the scale of the wavelength (0.8 μm). Several groups have studied SHG in biological tissues[4,5,6,7,8,9,10,11,12]. Of particular relevance to this work are several previous studies of the polarization dependence of the second harmonic signal in rat-tail tendon, performed over fifteen years ago[13,14,15].

A key problem with using SHG as a diagnostic tool in biological tissue has been that the absorption and scattering properties of tissue can affect the detected second harmonic signal intensity, introducing unwanted variability into the measurement that cannot be easily removed. In recent work, we described a technique that attempts to eliminate this problem by measuring the

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polarization dependence of the second harmonic signal[16]. The measured dependence of the signal on the input polarization direction can be fit to this model to determine a parameter we call γ which is directly related to the ratio of the two independent elements in the nonlinear susceptibility tensor. We found that, for the highly organized bundles of parallel fibrils in rat-tail tendon, the value of γ varies between about —0.7 and —0.9. However, the measurement of γ was time-consuming and tedious. At a single point on the sample, we measured the signal intensity at 36 different polarization angles from 0 to 360 degrees in order to have enough resolution to determine γ from the fit. In order to provide a clinically useful diagnostic tool, γ must be measured more quickly. In this work, we describe a new technique that uses an electro-optic modulator (EOM) to rotate the polarization and phase-sensitive detection at the first two harmonics of the modulation frequency in order to measure γ directly.

2. THEORY

For the cylindrical symmetry of bundles of parallel collagen fibrils, assuming Kleinman symmetry, the second order nonlinear polarization due to the electric field of the incoming laser beam $\vec{E}_1$ is,

$$\vec{P} = a\vec{s}\left(\vec{s} \cdot \vec{E}_1\right)^2 + b\vec{s}\left(\vec{E}_1 \cdot \vec{E}_1\right) + 2b\vec{E}_1\left(\vec{s} \cdot \vec{E}_1\right), \qquad (1)$$

where $\vec{s}$ is a unit vector in the direction of the fibrils and a and b are constants. Making the paraxial approximation and neglecting the effects of collagen birefringence on the linear optical properties of the tissue, the second harmonic signal is given by,

$$I_{SHG} = \frac{1}{4}\left(-1 - 6\gamma + \cos 2\alpha\right)^2 \sin^2\alpha + \cos^2\alpha\left(\gamma + \sin^2\alpha\right)^2, \qquad (2)$$

where $\gamma = b/a$ and α is the angle between the x-axis and the input beam polarization direction. The collagen fibrils are oriented along the y-axis; refer to Figure 1 for an illustration of this geometry. Physically, the parameter γ is related to the ratio between the polarizability along the axis of the fibril and the polarizability perpendicular to this axis. A detailed derivation of equation (2) is provided in our previous work [16].

3. EXPERIMENT

We used a Ti:Sapphire oscillator (SpectraPhysics Tsunami) to generate 5 nJ, 100 fs pulses at a wavelength of 800 nm and with a repetition rate of 82 MHz. The beam passed through an EOM (Conoptics Inc. 360-80) oriented with its axes at 45 degrees to the input polarization. A 3 kHz sawtooth wave was applied to the EOM with a peak-to-peak voltage designed to generate a 0 to 360 degree phase shift between the electric field components along the two modulator axes. A quarter-wave plate, oriented with one of its axes parallel to the initial laser beam polarization, was placed immediately after the EOM to produce linearly polarized light which sweeps from 0 to 180 degrees at the modulation frequency. The beam is reflected off a dichroic mirror and focused onto the sample through a microscope objective (Mitutoyo plano apochromat, f = 1 cm, N.A. = 0.42). The back-scattered second harmonic light is collected by the same objective, transmitted through the dichroic mirror, focused through a pinhole to reject out of focus light, and reflected onto a photo-multiplier tube (PMT) by a series of dichroic mirrors that reject the first harmonic light. The use of confocal microscopy allows measurements with a resolution of about 10 μm axially and 1 μm in the transverse direction. A diagram of our setup is given in Figure 1.

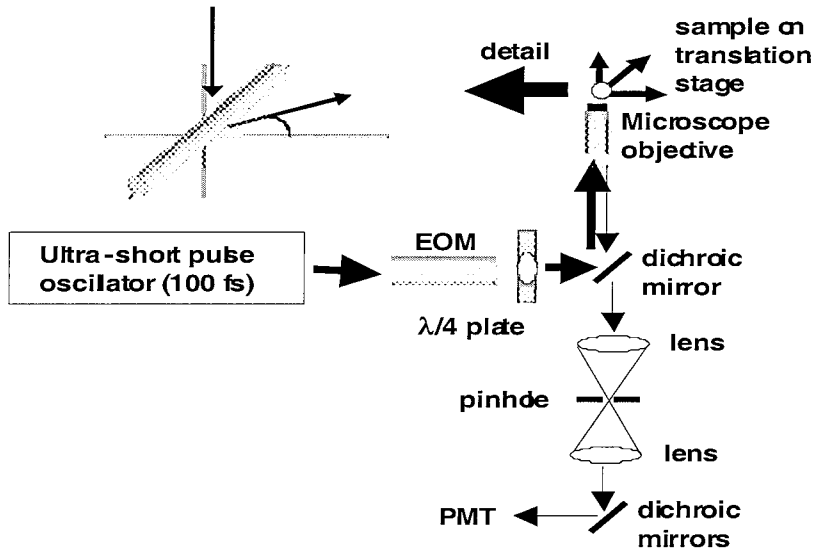

Figure 1. Illustration of the experimental setup used for polarization dependent scans, indicating the geometry of the incident laser beam polarization and collagen fibril bundle.

The signal from the PMT is detected using a lock-in amplifier that uses the sawtooth input to the EOM as its reference. Over a single period of the modulation ($0 < t < 2\pi/\Omega$), the polarization of the input laser beam can be written as $\alpha = \Omega t/2$, where Ω is the modulation frequency. The expression for the second harmonic signal becomes

$$I_{SHG} = \frac{1}{8}(3+20\gamma+40\gamma^2) - \frac{1}{2}(1+6\gamma+8\gamma^2)Cos(\Omega t) + \frac{1}{8}(1+4\gamma)Cos(2\Omega t), \qquad (3)$$

where we have simply substituted the expression for α into (2) and applied a few trigonometric identities. The ratio R between the signal at the second modulation harmonic and the first modulation harmonic is then

$$R = -\frac{1}{4+8\gamma} \qquad (4)$$

Solving for γ as a function of R, we have $\gamma = (-1-4R)/8R$. It is clear from a glance at Equation (3) above that the ratio, Q, of the signal at the third modulation harmonic to the signal at the first modulation harmonic should be zero. We have made the above calculations using the simplifying assumption that the collagen fibrils are oriented along the y-axis (refer to Figure 1); however, the result is identical when there is an arbitrary angle in the x-y plane between the fibrils and the y-axis. For the same reason, the absolute phase shift between field components polarized along the EOM axes has no effect on the result.

Using the lock-in amplifier phase measurement the phase shift between the reference signal and the detected signal at the first modulation harmonic, the relative orientation of different collagen fibril bundles can be determined. When the reference signal is at a maximum, the beam emerging from the quarter-wave plate is polarized along the y-axis. For the values of γ that we measure, the peak of the first modulation harmonic of the detected signal occurs when the polarization direction and the fascicle are parallel, or nearly parallel. Therefore, when the fascicle is oriented along the y-axis, the peak of the reference signal and the detected signal coincide there is no phase shift. When the fascicle is oriented at an angle θ from the y-axis, there is a 2θ phase shift between the two signals. Thus, this technique can be used to determine the relative orientation of different collagen fibril bundles.

4. RESULTS

We have tested the techniques described above using previously frozen, dry rat-tail tendon fascicles harvested from 350-gram Sprague-Dawley rats and gently compressed between two glass slides for mounting in the sample holder. The fascicles are a few centimeters long but only a few hundred micrometers in diameter. Figure 2 shows a one-dimensional axial scan of a rat-tail tendon fascicle. Each axial scan was performed twice while measuring the signal at the first, second, and third modulation harmonics; the two repeated scans were averaged. The repeated scans (not shown) confirm that the sample was not damaged by the focused laser beam. Figure 2 also shows the measured values for the ratios R and Q defined above. Using the formula for $\gamma\_R\_$ derived above, we obtain values forγ between -0.7 and -0.9. The Q ratio is non-zero due to the noise inherent in the measurement. We investigated several other fascicles from the same rat-tail and found that the mean γ was -1.17 with a standard deviation of 0.66; the median was -0.89. This is a larger range than what we observed in our previous study, probably due to the larger number of samples the faster technique allowed [16].

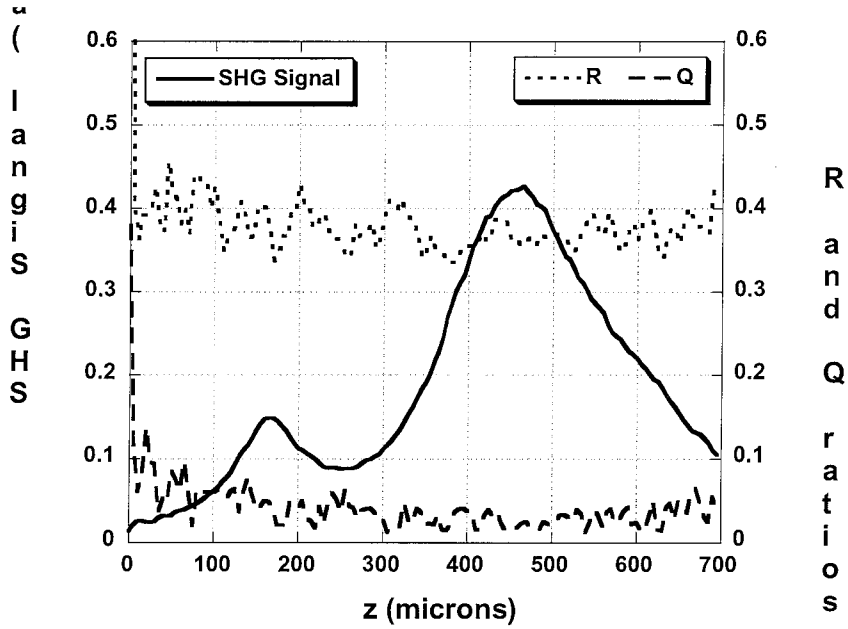

Figure 2. Plot of the second harmonic signal at the first modulation harmonic (FMH) and the ratios R and Q for a rat-tail tendon fascicle oriented as shown in Figure 1. Note that the signal strength varies by about an order of magnitude, while R varies only by about 20 %. The non-zero measured values of Q are due to noise.

We have used the phase measurement to determine the relative orientation of two fascicles that were oriented at approximately 90 degrees to each other, placed on top of each other, and compressed between glass slides. As shown in Figure 3, the phase measurement jumps by about 180 degrees as we scan from one fascicle to the other, as predicted.

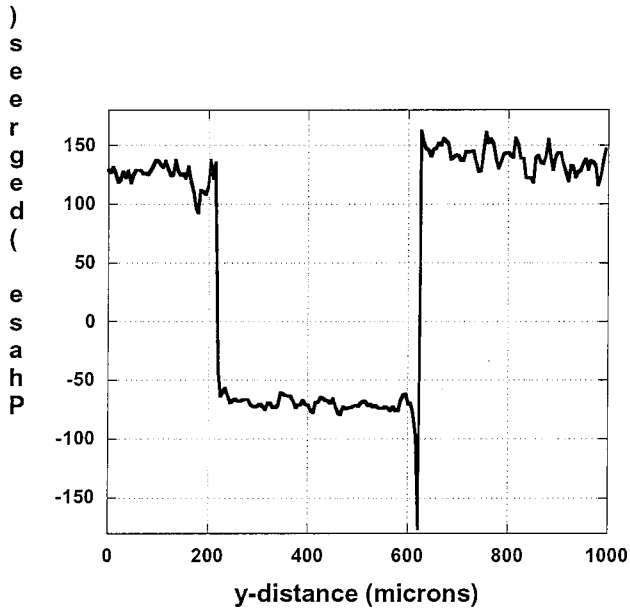

Figure 3. Plot of the phase of the second harmonic signal at the first modulation harmonic. The fascicle stretching along the y-axis is overlapped by a fascicle stretching along the x-axis between y = 200 μm and y = 600 μm. Note the ~180... change in phase as the beam is scanned from the first fascicle to a fascicle oriented at approximately 90... to it.

5. DISCUSSION

We have shown that linear polarization modulation, coupled with detection of the amplitude of the first and second modulation harmonics of the optical second harmonic signal can be used to measure γ, a parameter directly related to the ratio between the two independent elements in the second order nonlinear susceptibility tensor. Further, we have demonstrated that the orientation of collagen fibrils can be determined using the phase measurement at the first modulation harmonic.

In these initial measurements and calculations we have neglected the effect of the linear birefringence of the tissue. We have also not considered the possibility that scattering of light out of the beam as it propagates through the tissue and back-scattering of the second harmonic light by the parallel collagen fibers may itself be dependent on the polarization. In future work, we plan to investigate these effects and, if necessary, include them in our model of second harmonic generation in collagen.

Our initial measurements have used rat-tail tendon a much studied and highly organized tissue but we plan to apply this technique to collagen structures in other more heterogeneous tissues, such as skin. Changes in the molecular structure and supramolecular organization of collagen that affect its nonlinear susceptibility should be detectable using our technique. We plan to investigate the changes in collagen structure brought about by abnormal wound healing and by glyco-oxidative damage, for example. While such conditions would probably affect the amount of second harmonic signal generated, the amount of second harmonic light detected also depends strongly on the highly variable scattering and absorption properties of tissue, making direct comparison between healthy and unhealthy tissue very difficult.

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